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SCIENCE DIRECT

Virology 347 (2006) 109 – 116

VIROLOGY

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# Regulation of the expression of the Epstein–Barr virus early gene BFRF1

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Received 28 July 2005; returned to author for revision 28 September 2005; accepted 30 November 2005
Available online 9 January 2006

**Abstract**

The switch from latency to lytic phase of Epstein–Barr virus (EBV) is coordinated by the expression of two viral transactivators known as ZEBRA and RTA. The BFRF1 gene has been shown to be transcribed during the early phases of EBV lytic cycle. Here, we characterized the BFRF1 promoter showing that ZEBRA transfection stimulated BFRF1 expression, whereas RTA induced BFRF1 only after the transfection of an amount of plasmid largely in excess than that sufficient to stimulate the expression of other RTA-responsive genes. However, a co-operative effect between ZEBRA and RTA in the expression of BFRF1 is evident since the transfection of RTA can rescue the transactivating capacity of a mutant of the ZEBRA protein, known as Z(S186A), that has a substitution affecting the DNA binding region. Moreover, we identified one ZEBRA-responsive element (ZRE) and one RTA-responsive element (RRE) within the BFRF1 promoter region.


*Keywords:* Epstein–Barr virus; BFRF1 regulation; Lytic cycle

## Introduction

Epstein–Barr virus (EBV), a highly diffused gammaherpesvirus, is the etiologic agent of the infectious mononucleosis and has been associated to different malignancies of B lymphoid and epithelial origin such as Burkitt's lymphoma (BL), Hodgkin's disease, B and T cell lymphomas in immunocompromised individuals and nasopharyngeal carcinoma (Kieff and Rickinson, 2001). After primary contact, EBV establishes a life long infection, and the virus can be found in circulating B lymphocytes as well as in epithelial cells of the nasopharynx and the tongue. During this period, the viral infection is mainly kept in a latent state, characterized by the expression of a restricted subset of viral proteins. Under circumstances largely unknown, the EBV replicative cycle is reactivated, thus leading to a new viral progeny, which causes phases of viremia and viral shedding in the saliva.

Most of the knowledge on EBV biology is derived from B lymphoblastoid cell lines immortalized by the virus. In these cells, EBV establishes a latent infection, and the lytic cascade can be induced by the treatment of the cells with different stimuli. Two EBV proteins, translated from the BZLF1 and BRLF1 genes and indicated as ZEBRA and RTA respectively, have been shown to be the key regulators of the lytic cycle (Countryman and Miller, 1985; Zalani et al., 1997; Ragoczy et al., 1998, Feederle et al., 2000). These proteins act as transactivators by inducing directly or indirectly the expression of the lytic genes. About 30 EBV lytic proteins have been identified so far. They have been classified in early or late lytic proteins according to the susceptibility to be expressed in the presence of inhibitors of the EBV DNA polymerase: lytic genes whose expression is blocked by this treatment are classified as late genes, whereas early lytic genes are not affected (Serio et al., 1997). Alternatively, lytic genes have been distinguished in three groups according to their responsiveness to the two viral transactivators: a class directly activated by ZEBRA, a second class regulated by RTA and a third group whose expression is synergistically induced by ZEBRA + RTA (Ragoczy et al., 1998).

We have identified a novel EBV gene, BFRF1, that encodes a lytic protein of 37 kDa expressed in the early phases of the replicative cycle (Farina et al., 2000). The BFRF1 gene and

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doi:10.1016/j.virol.2005.11.046

more in general the entire region of EBV genome that encompasses this gene are strongly conserved among herpesviruses, thus suggesting that proteins encoded within this fragment play a role in biological functions that are pivotal to herpesviruses replication (Hudson et al., 1985). Indeed, it has been recently shown that the proteins generated by the viral genes BFRF1 and BFLF2 are co-operatively involved in the process of egress of the newly synthesized virions from the nuclei of infected cells (Lake and Hutt-Fletcher, 2004; Farina et al., 2005; Gonnella et al., 2005).

In the present study, we have studied the mechanisms of regulation of the BFRF1 gene in an attempt to add a further element to the knowledge of the EBV replicative program.

## Results and discussion

### *Characterization of the 5′-end of the BFRF1 transcript*

We initially mapped the start site of the BFRF1 mRNA by 5′-RACE using RNA isolated from the EBV-positive Raji cell line chemically induced to lytic cycle by treatment with TPA and butyrate. A reverse transcription reaction was performed on extracted RNA, and amplified cDNAs were purified and cloned to be fully sequenced.

The sequence of the longest 5′-clone generated showed that the transcription started at position 58,585 of the EBV B95-8 DNA sequence, at −306 bp from the BFRF1 ATG (genomic co-ordinates 58,891) and is reported in Fig. 1A. The sequence TTAATA located at position 58,563 (−22 bp from the identified transcription start) is likely to represent the TATA box of the BFRF1 gene.

Subsequently, studies were undertaken to evaluate the mechanisms that regulate the expression of the BFRF1 gene. We have previously observed that high levels of BFRF1 expression in EBV-infected cell lines are obtained after the combined treatment with TPA and butyrate. TPA-mediated activation of the EBV lytic cycle is known to be based on the capacity to drive the expression of the viral transactivator BZLF1. This is achieved through the activation of a complex of factors that by binding to specific DNA sequences known as TPA Responsive Elements (TREs) in the promoter region of responsive genes finally activate the transcription machinery (Flemington and Speck, 1990; Liu et al., 1997). In the case of EBV, the ZEBRA protein is subsequently phosphorylated and acts as BRLF1 transactivator, thus leading to the co-operative induction of the viral replicative cycle (Baumann et al., 1998; Feederle et al., 2000; El-Guindy and Miller, 2004).

On the basis of the mapping of BFRF1 transcription start, we performed a computer analysis of the region upstream the BFRF1 ATG, searching for known TPA (TRE)-, ZEBRA (ZRE)- or RTA (RRE)-responsive consensus motifs, and the results are summarized in Fig. 1B. No TRE is present in the BFRF1 promoter region, whereas a canonical ZRE and one putative RRE, similar to the RRE-DR1 elsewhere described (Gruffat and Sergeant, 1994), are present at positions 58,373 and 58,518 of the B95-8 EBV strain, with respect to the translation start located at 58,891. In detail, a ZIIIA motif (TGAGCCA) at −373 bp from the ATG and a putative RRE

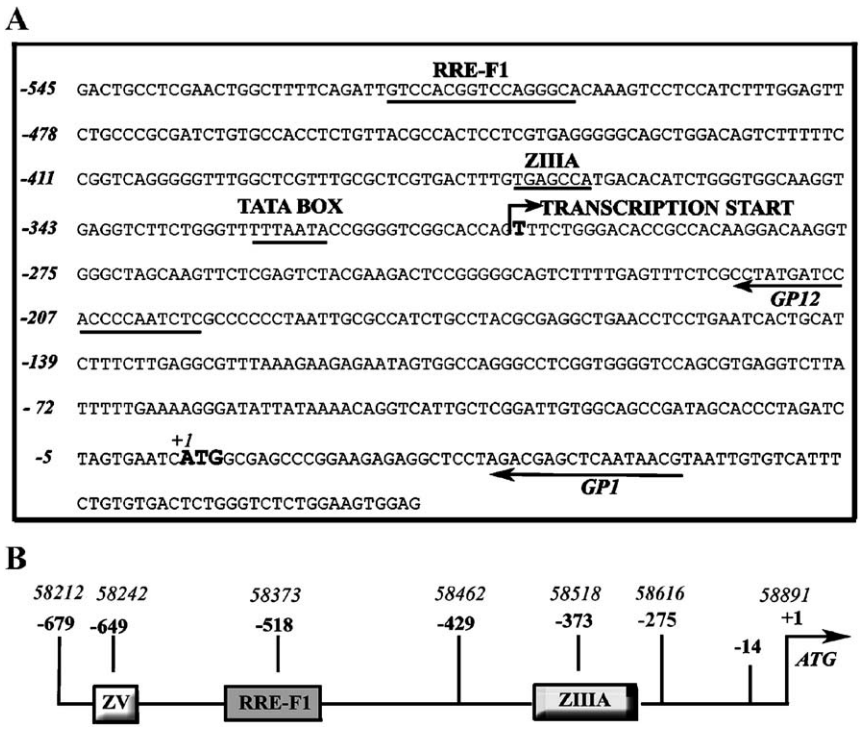


Fig. 1. (A) Sequence of the promoter region of the BFRF1 gene and mapping of the transcription start site of BFRF1 determined by 5′-RACE. The transcription start (bent arrow) and the position of the primers used for the 5′-RACE (leftward arrows) are indicated. The positions of the potential RRE, ZRE and TATA box are underlined. All the positions are calculated relative to the BFRF1 ATG (bold characters). (B) Schematic diagram indicating the positions of the potential ZEBRA- and RTA-responsive elements (ZIIIA and RRE-F1, respectively) and the negative regulator consensus sequence (ZV) relative to the BFRF1 ATG.

motif (GTCCacggtccagGGCA), herein indicated as RRE-F1, positioned at −518 bp, were found by the computer analysis (Fig. 1B). The RRE-F1 motif is not a canonical RRE since the consensus sequence usually found is GNCCN$^9$GGNG, however, it is similar in the 3′-core to an RRE present within the EBV BHLF1 promoter (indicated as BHLF1-I) recently described by other authors, which has been shown to be very active in responding to RTA expression (Chen et al., 2005).

### *In EBV-infected cell lines, BFRF1 activation is mainly regulated by ZEBRA expression*

In EBV-infected cell lines, the switch from latency to replication is driven by either ZEBRA or RTA. In epithelial as well as in some permissive B lymphoblastoid cell lines, the transfection of either ZEBRA or RTA genes triggers the expression of the other immediate-early factor, and, together, these proteins act individually or in synergy on downstream targets to activate the viral lytic cascade (Ragoczy et al., 1998). In other B cell lines, such as Raji cells, RTA transfection is not sufficient to stimulate the full viral cascade, and only a subset of early proteins are expressed.

We set up transfection experiments on Raji cells to determine the responsiveness of the BFRF1 gene to the exogenous expression of either ZEBRA and/or RTA. We also took advantage of a plasmid expressing a mutant form of the ZEBRA protein, known as Z(S186A): this mutant has a substitution of the serine located at position 186, which is phosphorylated in TPA-treated cells, with an alanine residue (Francis et al., 1997; El-Guindy et al., 2002). More important, it has been observed that the Z(S186A) mutant has lost the ability to induce the lytic cycle, although still maintaining the capacity to co-operate with RTA and transactivate specific promoters (Francis et al., 1999; Bhende et al., 2005).

Raji cells were electroporated with plasmid expressing either RTA, ZEBRA or Z(S186A), and protein extracts were analyzed by Western blot to detect the expression of four viral lytic gene products: ZEBRA, RTA, BFRF1 and EA-D, the BMFR1-encoded protein.

As shown in Fig. 2A, all the four lytic proteins were easily detected following ZEBRA transfection as well as in co-transfection with RTA. On the other hand, RTA was able to induce EA-D expression but not BFRF1 and BZLF1, as also observed in the same cell line by other authors (Ragoczy and Miller, 1999). Furthermore, the Z(S186A) mutant failed to induce both EA-D and BFRF1. However, BFRF1 and EA-D expressions were strongly detected when RTA was co-transfected with Z(S186A). Interestingly, as also observed by other authors, the transactivating capacity of RTA is affected by the amount of the plasmid transfected. Indeed, increasing amounts of RTA (5 and 10 μg, respectively) caused a higher level of EA-D expression and the detection of a signal of

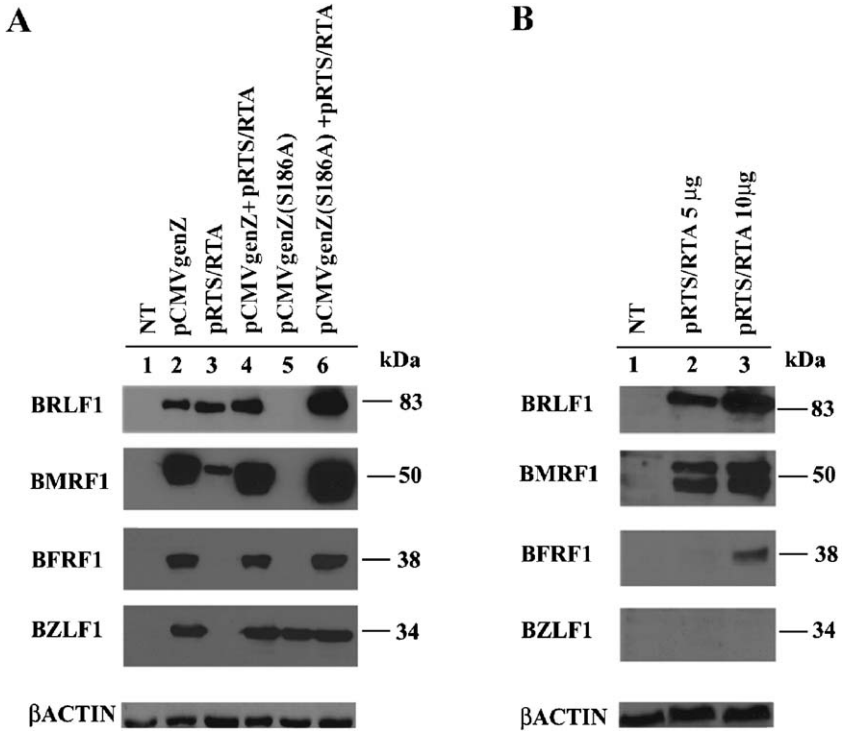


Fig. 2. Expression of EBV lytic proteins in transfected Raji cell. (A) Western blot analysis showing the expression of BRLF1, BMRF1, BFRF1 and BZLF1 proteins after transfection of either pCMVgenZ, pRTS/RTA alone or in combination and pCMVgenZ(S186A) alone or in combination with pRTS/RTA. Lane 1: untransfected Raji cells; lane 2: transfected with 10 μg of pCMVgenZ; lane 3: transfected with 1 μg of pRTS/RTA; lane 4: transfected with 10 μg of pCMVgenZ and 1 μg of pRTS/RTA; lane 5: 10 μg of pCMVgenZ(S186A); lane 6: transfected with 10 μg of pCMVgenZ(S186A) and 1 μg of pRTS/RTA. Cell lysates were collected after 48 h after transfection and assayed by monoclonal antibodies. Equal loading was assessed by probing the blot with monoclonal antibodies to β-Actin. (B) Western blots showing that increased amounts of exogenous RTA induce a weak BFRF1 expression. Lane 1: untransfected Raji cells; lane 2: transfected with 5 μg of pRTS/RTA; lane 3: transfected with 10 μg of pRTS/RTA. Cell extracts collection and equal loading as described in (A).

BFRF1 not visible with 1 μg of transfected pRTS15/RTA that is sufficient to induce EA-D transactivation (Fig. 2B). However, we tend to consider that usage of high amounts of exogenous proteins could influence the molecular stoichiometry, thus rendering the effects less stringent with the physiological conditions. Overall, these data seem to indicate that, in EBV-positive B cell lines, BFRF1 expression is mainly responsive to ZEBRA, but a role is certainly played also by RTA given the capacity to rescue the BFRF1 transactivation of the ZEBRA mutant Z(S186A).

*Identification of two consensus sequences responsive to ZEBRA and RTA within the BFRF1 promoter*

The presence of two potential ZEBRA- and RTA-responsive elements in the 5′-region of the BFRF1 promoter and the results of the previous set of experiments led us to analyze in detail the role played by the consensus sequences highlighted by the computer analysis. Therefore, we set up experiments of luc-assay using different subfragments of the 5′-region of BFRF1 gene: the −679pF1 and −429pF1 that contain the wild type sequence of the EBV genome and encompassing both the RRE-F1 and ZIIIA or only the ZIIIA motifs, respectively. We also assayed the constructs −679pF1-ZIIIA(c,a) and −429pF1-ZIIIA(c,a) containing a mutated ZIIIA domain as well as the −679pF1RREΔ mutant with deletions in the consensus sequence of the RRE-F1 domain. Finally, we generated a subfragment indicated as −275pF1, which does not contain neither the RRE-F1 nor the ZIIIA motifs (Figs. 3A and B).

The constructs so generated were transfected in an EBV-negative epithelial cell line, known as 293 cells, in the presence of either ZEBRA or RTA expressing plasmids; the luciferase activity was assayed 48 h post-transfection. As described in Fig. 4A, RTA was able to drive the transactivation of the −679pF1 promoter more efficiently than ZEBRA alone, however, only a slight increase rather than a synergistic effect was observed when both RTA and ZEBRA were co-transfected. In −429pF1, ZEBRA-induced transactivation was higher (13.3×), whereas activation by RTA was significantly reduced

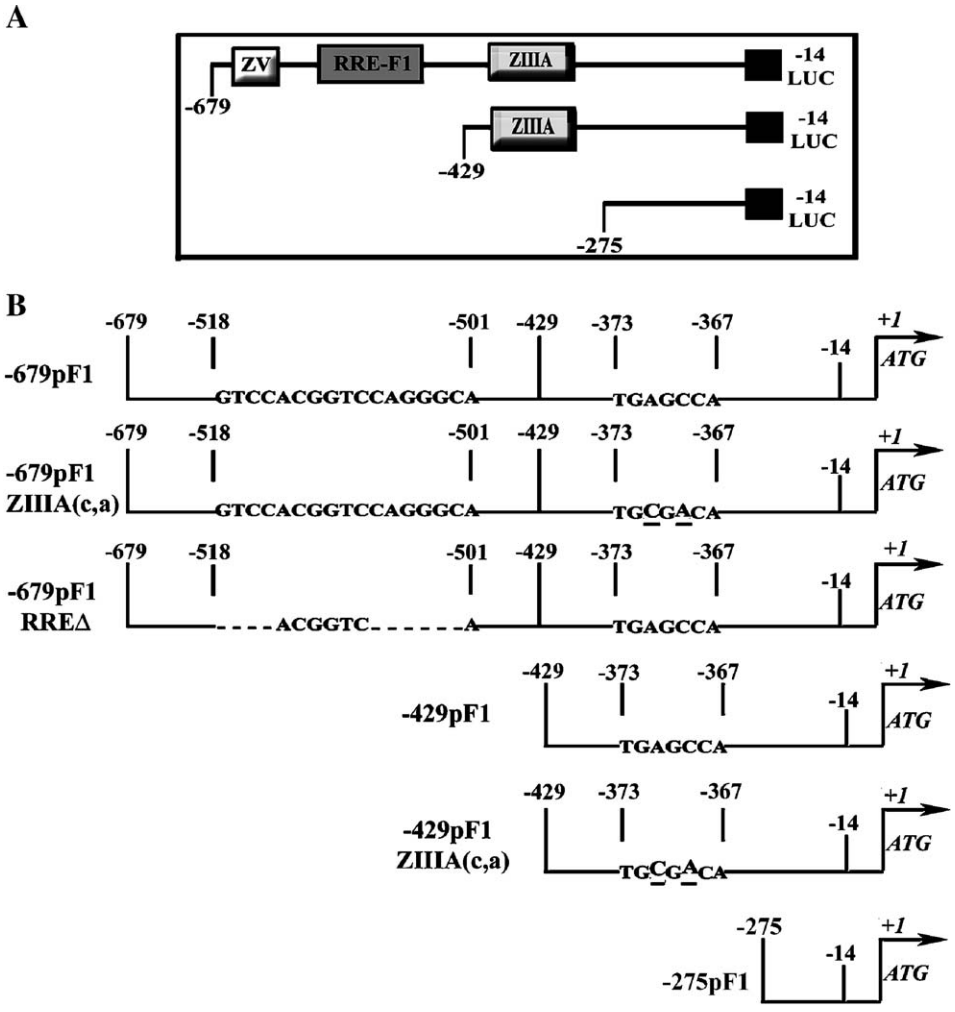


Fig. 3. Schematic diagram of the constructs used in luc-assays to characterize the BFRF1 promoter. (A) Map showing the presence or absence of the ZV, RRE-F1 and ZIIIA motifs in the constructs −679pF1, −429pF1 and −275pF1. (B) Sequence mutations in the plasmids used for the luciferase assays to inactivate the ZIIIA or the RRE-F1 consensus motifs. +1 indicates the BFRF1 ATG. The mutated bases within the ZIIIA motif are underlined. The bases deleted to inactivate the RRE-F1 are underscored.

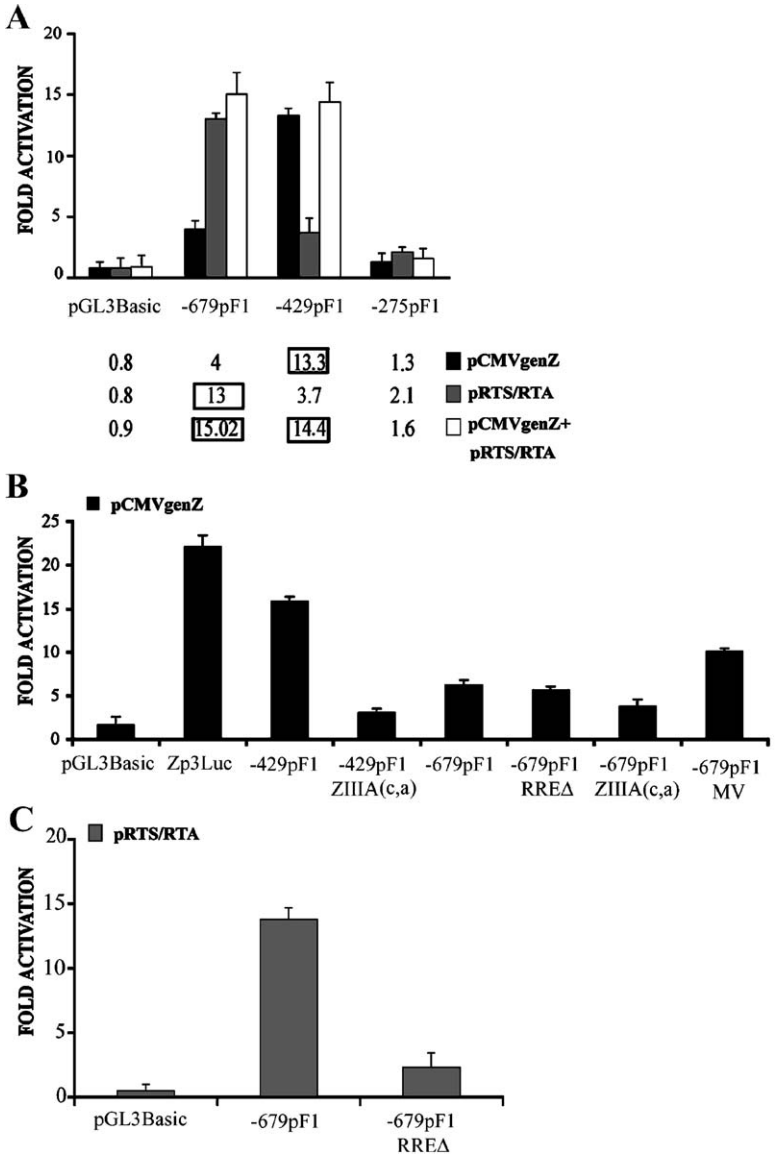


Fig. 4. Analysis of the BFRF1 promoter region. EBV-negative 293 cells were co-transfected with reporter plasmids and pCMVgenZ, pRTS/RTA Bars represent the mean of three experiments. Error bars show the standard deviation. Folds of activation are calculated relative to the transfection of control vector. As experimental controls, the promoterless reporter plasmid pGL3Basic and the Zp3Luc, bearing the BZLF1 promoter responsive to ZEBRA, were used. (A) 293 cells were transfected with 0.5 μg of −679pF1, −429pF1 or −275pF1 in the presence of 0.5 μg of pCMVgenZ or 0.2 μg pRTS/RTA alone or in combination. Fold of activation of each transfection experiment is indicated under the graphic. The boxes highlight the higher fold of activations observed. (B) 293 cells were transiently transfected with 0.5 μg of −679pF1, −429pF1 (wild type); −679pF1 ZIIIA (c, a), −429pF1 ZIIIA (c, a) (containing a mutation in ZRE element); −679pF1 RREΔ (containing a deletion of RRE-F1-responsive element); −679pF1MV mutant (containing a point mutation of ZV site). The reporter plasmids were co-transfected in combination with 0.5 μg of pCMVgenZ. (C) Transfections were performed using 0.5 μg of −679pF1 and −679pF1 RREΔ. In the same experiments, the reporter plasmids were co-transfected with 0.2 μg pRTS/RTA.

compared to the −679pF1 plasmid (by 3.5-fold), conceivably due to the lack of the RRE-F1 motif. Also, in this case, no synergistic transactivation was detected when both RTA and ZEBRA were co-transfected. Finally, no significant stimulation by either ZEBRA or RTA was observed in the −275pF1 reporter plasmid. This set of experiments suggested that the RRE-F1 and ZIIIA elements present in the analysis of BFRF1 promoter region are likely to be involved in the regulation of BFRF1 expression, although a more precise analysis was required to confirm this finding. The absence of a real synergistic effect by ZEBRA and RTA in stimulating the activity of the reporter plasmids here used was rather unexpected. A potential explanation might be the relative weakness of the BFRF1 promoter-derived constructs to be induced, as observed in all the series of experiments: in this case, it is possible that the mono transfections of RTA and ZEBRA were able to stimulate the −679pF1 and −429pF1 reporter plasmids, respectively, near to the plateau level which can be achieved by the BFRF1 promoter. Transfection of reduced amounts of pCMVgenZ and pRTS/RTA in the presence of the above mentioned reporter plasmids failed in inducing significant transactivation, thus preventing us from analyzing a potential synergism by lowering the scale of the response.

In the next series of experiments, we analyzed the capacity of the ZIIIA and RRE-F1 motifs to act as responsive elements for ZEBRA and RTA. As shown in Fig. 4B, ZEBRA induces the activation of the control plasmid bearing the BZLF1 promoter, already known to be responsive (22×) and is also an efficient transactivator of the −429pF1 plasmid (15.8×). The mutation of the ZIIIA site within this construct heavily affected the transactivation capacity of ZEBRA, reducing by more than 5-fold compared to the −429pF1 (3× of activation above background). Furthermore, the transactivation by ZEBRA was not so high when we used the −679pF1 construct, which retains the ZIIIA and the RRE elements (6.2× activation). The mutagenesis of the ZIIIA consensus motif reduced the ZEBRA-mediated transactivation of the construct, whereas the mutation of the RRE-F1 was almost ineffective (3.7× and 5.6× respectively), compared to the wild type −679pF1.

The finding of the low transactivation of the plasmid bearing the longer form of the promoter region led us to hypothesize the existence in the region between −429 and −679 of a repressor element that might interfere with the ZEBRA-mediated induction. The search for known repressor motifs by computer analysis indicated the presence in the −679 fragment of a sequence called ZV (5′-CAGGTG-3′) also identified in the promoter of BZLF1 gene (Fig. 1B) (Kraus et al., 2001). This site is recognized by a factor indicated as ZEB (Kraus et al., 2003). To assess the role of this sequence in the activation of −679pF1 reporter plasmid by ZEBRA, we introduced the same single base substitution (5′-CAGGTc-3′) in the ZV site described in the abovementioned report. Thus, the −679pF1MV mutant was generated, and we observed that the luciferase activity of this reporter in the presence of ZEBRA was increased (10×) when compared to that of the wild type −679pF1 construct (6.2×) as shown in Fig. 3A, although the mutation in the putative ZV site was not sufficient to restore the same level of transactivation observed in the −429pF1 plasmid. It should be noticed however that the relative position of the ZV domain in the BFRF1 promoter is upstream with respect to the ZIIIA element, whereas for example in BZLF1 promoter, its position is downstream than that of the known ZIIIA/ZIIIB and ZII sites that bind activators (Kraus et al., 2001). It is possible that the relative position of these elements might affect the repressor function of the ZV motif.

In the same set of experiments, we also assayed the capacity of RTA to transactivate the −679pF1 plasmid, which bears the RRE-F1 domain. As shown in Fig. 3C, RTA transactivates the reporter plasmid (14.8× over background), whereas the deletion of the RRE-F1 region lowered by 6-fold the responsiveness of the assay (2.5×). The capacity of RTA to induce the −679pF1 plasmid is as good as that observed for ZEBRA to transactivate the −429pF1 construct, although in Raji cells RTA alone failed to induce BFRF1 induction, unless we transfected high amount of pRTS/RTA plasmid. Some hypotheses could explain this discrepancy: epigenetic factors could influence the responsiveness of the BFRF1 promoter to ZEBRA and RTA in the context of the whole EBV genome. Alternatively, motifs located 5′- or 3′-promoter fragments cloned in the −429pF1 and −679pF1 constructs might be capable to influence the response to the two viral transactivators in vivo.

It should be noticed that all the luc-assays were also conducted in the EBV-negative B cell line, DG75, confirming the results observed in the epithelial cells. Taken together, the results of the assays indicate that in the context of a reporter plasmid the canonical ZIIIA and the novel RRE-F1 elements identified by the computer analysis represent the motifs that provide the responsiveness to ZEBRA and RTA, respectively.

In conclusion, we have characterized the transcription start of the BFRF1 mRNA by 5′-RACE. The results here described indicated that BFRF1, encoding a protein which is part of the early phase of the replicative cycle, can be classified as a gene directly transactivated by ZEBRA and RTA, the immediate-early proteins of EBV, with the former being more effective in stimulating BFRF1 expression in EBV-infected cells. Furthermore, the observation that RTA can rescue the capacity of Z(S186A) in transactivating BFRF1 adds a new tool for studies aimed to better define the mechanisms that lead to the EBV replicative cycle. In previous reports, a new classification of EBV lytic genes based on the responsiveness to RTA or ZEBRA alone or in combination has been proposed (Ragoczy and Miller, 1999): the results of the present assays indicate that BFRF1 gene can be included in this latter group.

## Materials and methods

### *Cell lines*

DG75 is an EBV-negative human B-cell line derived from a BL (Ben-Bassat et al., 1977). Raji is a human B cell line derived from a BL containing an EBV strain defective in viral replication (Decaussin et al., 1995). 293 is a human embryonic epithelial kidney cell line (Graham et al., 1977). All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. EBV chemical induction on Raji cells was achieved by treatment for 48 h with either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) 20 ng $ml^{-1}$ and 3 mM of sodium butyrate.

### *5′-RACE*

To study the transcription start site of BFRF1 gene, 5′-RACE was performed with RNA isolated from Raji cell line induced by TPA/butyrate treatment. Total RNA was extracted with TRIzol (Life Technologies). Reverse transcription and PCR were carried out using the 5′-RACE kit (Roche) in the presence or absence of reverse transcriptase as control. For the reverse transcription, a primer designated as GP1 (CGTTATTGAGCTCGTCT, coordinates 58,934–58,917) was used. For the subsequent 5′-RACE, PCR was synthesized an oligonucleotide indicated as GP12 (GAGATTGGGGTGGATCATAGG, coordinates 58,691–58,670) and the Oligo dT-Anchor primer provided by the kit. Amplified cDNAs were separated on 2%

agarose gel, purified and subsequently cloned into the pGEM-T Easy Vector (Promega). DNA from isolated clones was then extracted and fully sequenced.

*Plasmids construction*

For luciferase assays, subfragments of the region upstream to the ATG of BFRF1 were cloned into the backbone of the pGL3Basic vector (Promega). −679pF1 was obtained by cloning the 665 *Drd*I/*Bgl*II fragment (coordinates 58,212–58,877) into *Sma*I/*Bgl*II sites of the vector. −429pF1 was generated by cloning the 415 *Pvu*II/*Bgl*II fragment (coordinates 58,462–58,877) into *Sma*I site of the vector. To generate the −p275F1 plasmid, we first subcloned a 746 *Bst*xI/*Bgl*II fragment (genomic coordinates 58,131 to 58,877) into *Sma*I/*Bgl*II sites of pGL3Basic vector (Promega) and then excited a 390 bp using *Nhe*I restriction enzyme. This construct encompasses EBV genomic coordinates 58,616 to 58,877. To generate the Zp3Luc plasmid, the 561 bp *Bam*HI/*Nae*I fragment from the *Bam*HI Z region was cloned into the *Sma*I site of pGL3Basic vector (coordinates 103,742–103,182). pRTS/RTA containing the BRLF1 gene, pCMVgenZ containing the BZLF1 gene and pCMVgenZ(S186A) containing a mutant of the BZLF1 gene were kindly provided by Dr. George Miller (Yale University). The pRL-TK (Promega) was used as an internal control plasmid expressing *Renilla* luciferase driven by the thymidine kinase promoter of herpes simplex virus.

*Site-directed mutagenesis*

We performed a site-directed mutagenesis of the ZIIIA and RRE-F1-responsive element of the BFRF1 promoter using the Exsite PCR-Based site-directed mutagenesis kit (Stratagene). The −429pF1 ZIIIA (c,a) and −679pF1 ZIIIA (c,a) mutant containing the ZIIIA were generated by two PCR reactions using the primers 1C429 (5′-**TGcGCCA**TGACACATCTGGGTGG-3′) (58,518–58,541) and 1A429 (5′-**TGcGaCA**TGACACATCTGGGTGG-3′ (58,518–58,541) as forward oligos coupled to the 2C429 (5′-CAAAGTCACGAGCGCAAACGAGC-3′ (58,517–58,494) as reverse oligo. The deletions in the RTA binding site of BFRF1 promoter were generated in the wild type vector −679pF1 using the kit described previously and the following primers: 1AD679 (5′-TCACAAAGTCCTCCATCTTTGG-3′) (58,381–58,409) as forward oligo coupled to 2D679 (5′-CCGTGGACAATCTGAAAAGC-3′ (58,380–58,360) and 14D666 (5′-CCGTAATCTGAAAAGCCAGT-3′) (58,380–58,356) as reverse oligos. All mutants obtained were fully sequenced.

*Cell transfections*

$10^7$ Raji cells or DG75 cells were electroporated using a Biorad Gene Pulser (0.26 kV and 960 μF) with appropriate amounts of DNA. For luciferase studies, the 293 cells grown in 12 or 24 multiwell were transfected by using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. 293 cells were transiently transfected with 0.5 μg of luciferase constructs, 0.5 μg pCMVgenZ and 0.2 μg of pRTS15/RTA.

*Protein extracts and Western blots*

Cells were collected by centrifugation, washed in PBS, lysed by sonication and suspended in 3× SDS sample buffer (Sambrook et al., 1989). For Western blot analysis, the samples were heated at 100°C for 5 min and loaded on a 12% SDS-PAGE. After electrophoresis, the gels were transferred onto a nitrocellulose membrane according to standard procedures (Sambrook et al., 1989). Membranes were first blocked 1 h at RT with blocking solution (1× PBS, 0.1% Tween-20 and 5% milk) and then incubated 1 h RT with the primary antibody, washed three times with washing solution (1× PBS, 0.1% Tween-20) and incubated 45 min RT with anti-mouse or anti-rabbit HRP-conjugated antibodies (SIGMA) at a dilution of 1:5000 in blocking solution. After further washes, proteins were detected by enhanced chemiluminescence according to manufacturer's instructions (Roche) and visualized on X-ray films.

*Reporter gene assay*

Luciferase assays were performed at 24–48 h post-transfection. 293 cells were harvested, washed twice with phosphate-buffered saline and suspended in 150 μl of lysis buffer (Promega) for 15 min at room temperature. Luciferase activities were determined using the Dual-Luciferase assay system according to the manufacturer's instructions (Promega). The pRL-TK (Promega) was used as an internal control plasmid. The activity of *Renilla* was used to calibrate the activity of firefly luciferase. The activity of the reporter plasmid was calculated and shown as fold activation. All the results of the reporter assays were based on experiments performed at least in triplicate.

## Acknowledgments

This work was partially supported by grants from MIUR-COFIN 2003; from Associazione Italiana per la Ricerca sul Cancro (AIRC); from Ministero della Sanità-Progetto AIDS. We are grateful to Prof. George Miller, Yale University for providing us the pCMV-genZ, pRTS/RTA and CMV-Z(S186A) plasmids.

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